Note on the Small Cloud.—It has been suggested that the Small Magellanic Cloud is also a quasi-barred spiral like the Large Cloud and that its elongation is an indication of its tilt. The variable stars provide no supporting evidence. For 211 cepheid variables in five areas outside the central body of the Small Cloud, we have assembled the mean systematic deviations of the observed median magnitudes from the adopted period-magnitude curve, as shown in the accompanying tabulation.

Area	Position	No. of Variables	Systematic Deviation
A	Southwest	37	$0^m.00 \pm 0^m.03$
B	Northeast	55	$-.03 \pm .03$
C	Southeast	41	$-.08 \pm .04$
D	East	41	$+.01 \pm .04$
E	Northwest	37	-0.17 ± 0.03

Summary.—The shape of the Large Cloud of Magellan, whether flattened or roughly spherical, remains undetermined. So far the evidence favors a chaotic form, but future studies with radio telescopes and possibly with stellar spectroscopes may clarify the matter. Our examination of the magnitudes of several hundred variable stars (38 of which were newly discovered in the course of the work) gives no positive evidence that these stars lie along a plane; but the criterion is not sensitive. A similar examination for the Small Cloud, involving 211 cepheids, is also inconclusive.

A by-product of the work is the evidence that a considerable amount of dusty obscuration affects the bar of the Large Cloud. Another by-product is an indication that the scatter in median magnitudes along the period-magnitude curves is not due to photometric inaccuracies but rather is attributable to other factors, including thickness of the Clouds in the line of sight, localized obscurations, and inherent dispersions in the median luminosities of classical cepheids.

[1] These PROCEEDINGS, **41**, 185–190, 1955; *Harvard Reprints*, No. 407.
[2] These PROCEEDINGS, **38**, 281–289, 1952; *Harvard Reprints*, Nos. 359 and 360.
[3] *Observatory*, **74**, 26, 1954.

ED

*RECONSTITUTION OF ACTIVE TOBACCO MOSAIC VIRUS FROM ITS INACTIVE PROTEIN AND NUCLEIC ACID COMPONENTS**

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Much recent evidence from chemical, physicochemical, electron microscopical, and X-ray studies has resulted in a definite concept of the structure of the tobacco mosaic virus (TMV) particle.[1–5] It appears that about 2,800 protein subunits of a molecular weight near 18,000 are arranged in a helical manner to form a rod with a hollow core. The nucleic acid is believed to occur as strands in the core. Electron micrographs which support this concept have been obtained of the virus at various stages of disaggregation.[3–5] A protein isolated from infected plants has been found to reaggregate—first to short pieces of the presumed helix lying on end and

resembling disks with central holes and then to much longer, but inactive, rods of the diameter of the virus yet free from nucleic acid.[6] It has now been possible to achieve the co-aggregation of inactive virus protein subunits and inactive virus nucleic acid to give nucleoprotein rods which appear to be infective.

Preparation of Protein and Nucleic Acid Components.—TMV was dialyzed against pH 10–10.5 glycine buffer (0.01 *M*) or pH 10.5 carbonate-bicarbonate (0.1 *M*) at 3° C. for 48–72 hours. Undegraded virus was separated by cold ultracentrifugation, and the supernatant was brought to 0.4 saturation with ammonium sulfate. The protein alone precipitates (optical density$_{260\ m\mu}$/optical density$_{280\ m\mu}$ = R = 0.65), leaving only nucleic acid (R = 2.0) in the supernatant; if this separation is not clean, longer alkali treatment is necessary. The protein moiety is precipitated twice more with 0.25–0.35 saturated ammonium sulfate, dialyzed, brought to pH 7.0–8.0 with NaOH, and finally again freed from heavy particles, such as undegraded virus, by ultracentrifugation. The protein gives a water-clear solution at pH 7; the masked —SH group is still present. The spectrum resembles that of a mixture of tryptophan, tyrosine, cysteine, and phenylalanine, simulating the composition of the protein, although the minimum (at 250 mμ) is not quite as low (max./min. = 2.4 versus 2.9) (Fig. 1); P analyses (0.01–0.03 per cent) indicate removal of about 95–98 per cent of the nucleic acid. Evidence for the absence of detectable virus particles will be discussed below.

The nucleic acid fractions from such alkali-degraded TMV are not as effective for reconstitution as that obtained by the detergent method.[2] A virus solution (1 per cent) containing 1 per cent sodium dodecyl sulfate is adjusted to pH 8.5 and held at 40° for 16–20 hours. Following this treatment, ammonium sulfate is added to 0.35 saturation, and the protein precipitate is separated by centrifugation. When the supernate is refrigerated, from 60 to 90 per cent of the nucleic acid precipitates (R = 2.0) and is centrifuged off the next day; it is further purified by repeated resolution in ice water and precipitation with two volumes of cold ethanol and a few drops of 3 *M* pH 5 acetate. The nucleic acid solution is finally subjected to cold ultracentrifugation to remove any traces of virus. The virtual absence of protein is indicated by a minimum in O.D. near 230 mμ (max./min. = 3.0) (Fig. 1).

In a few preliminary ultracentrifuge experiments, kindly performed by Dr. Howard K. Schachman, the nucleic acid preparations exhibited one principal boundary with a sedimentation coefficient of about 8 S, resembling the preparation of Cohen and Stanley.[7] The protein, in pH 9 borate (0.01 *M*) or in 0.01 *M* NaCl at that pH, exhibited largely a single boundary with a sedimentation coefficient of 4.5 S. At lower pH values larger components, presumably due to aggregation, were observed.

Reconstitution of Active Virus.—For reconstitution of the virus, 1 ml. of approximately 1 per cent protein solution is mixed with 0.1 ml. of a 1 per cent nucleic acid solution. Opalescence appears after addition of a suitable buffer; 0.01 ml. of pH 6 acetate (3 *M*) has given the best results, but phosphate (pH 6.3 and 7.0) and pH 6 ammonium acetate have also been used successfully. The samples are held at 3° for at least 24 hours. They may then be directly diluted and assayed. More often they were ultracentrifuged, the pellets redissolved in water, traces of insoluble material separated by centrifugation, and aliquots of the opalescent supernatants diluted for spectrophotometry. Most of the protein and 40–60

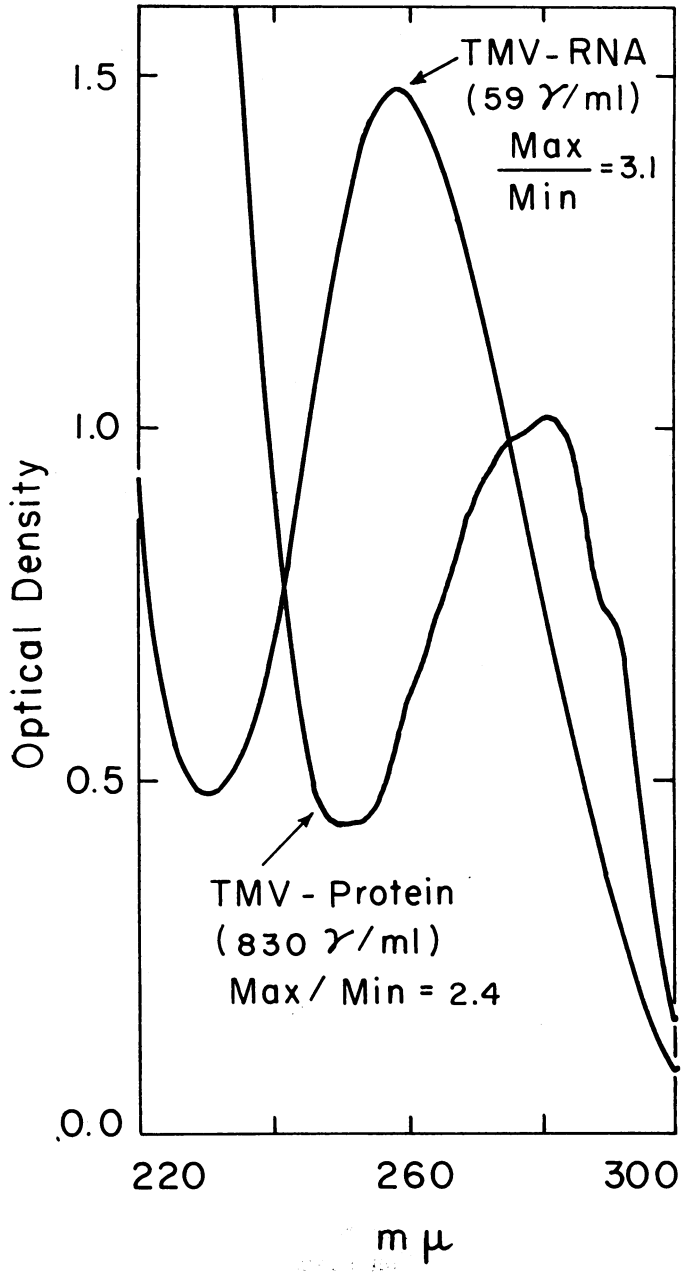

FIG. 1.—Ultraviolet absorption spectrum of purified virus protein and nucleic acid.

per cent of the nucleic acid[8] were in the pellet, and the spectrum was that expected for a nucleoprotein such as TMV (max./min. = about 1.17). On the basis of the O.D. of TMV (0.27 at 260 mμ for an 0.01 per cent solution), approximate concentrations were calculated, and the sample was diluted to a range of 100 to 10 μg/ml. for assay. For pellets composed of protein only (absorption maximum at 280 mμ),

calculations were based on the O.D. of the virus proteins (0.13 for an 0.01 per cent solution at that wave length). Assays were performed on groups of 8–12 plants (*Nicotiana glutinosa*), distributing each of an equal number of samples (including at least one standard and often a solvent blank) over 10 equivalent half-leaves. It was indeed quite surprising to find that the reconstituted nucleoprotein preparations produced local lesions (from 2 to 30 per half-leaf), when tested at 10–100 μg/ml., which were indistinguishable in appearance from those of TMV at 0.1 μg/ml. In contrast, no activity was observed when the protein or nucleic acid alone was tested up to 1,800 and 52 μg/ml., respectively.[9] When 0.005 per cent TMV was held for several hours in 5 per cent protein or 0.5 per cent nucleic acid, it was found fully active upon dilution and assay. This strongly suggests that the noninfectivity of the two components cannot be attributed to inhibition phenomena (Table 1).

TABLE 1

ACTIVITY OF THREE PREPARATIONS OF RECONSTITUTED VIRUS AND OF TMV AT DIFFERENT ASSAY LEVELS; ABSENCE OF INHIBITORS FROM COMPONENTS

MATERIAL TESTED	ASSAYS	
	μg./Ml.	Lesions/Half-Leaf*
Reconstituted virus:		
25,000-rpm. pellet	100	27 54 31 120†
	30	18 — —
	25	— 35 —
	10	9 7 14†
40,000-rpm. pellet	100	2 23 —
TMV stock preparation	0.25	27 15
	0.1	9 (6.1–12.1)‡
	0.02	5
TMV-protein added at 1000:1	0.1	10
TMV-RNA added at 100:1	0.1	5 13

* Each figure is the average of 10 equivalent half-leaves. The 3 columns in top half of table refer to separate preparations.

† This is one of the preparations which appeared to increase in activity during storage.

‡ Average and range of 9 assays (10 half-leaves each) of one TMV preparation over 3 months.

When each of the two components was diluted for assay to 0.2 and 0.02 mg./ml., respectively, prior to mixing, no activity was generated. Also, when the complete reaction mixture (5–10 mg./ml.) was diluted to 0.1 mg./ml. one minute after addition of the buffer which triggers the aggregation, no activity was obtained. About one hour at room temperature appears to be required for the formation of any active rods. These experiments represent convincing control data; they also seem to prove that we are dealing with a definite chemical reaction and to exclude most other interpretations. The observation that the nucleic acid gradually loses most of its activity during several weeks of storage at 3° (in aqueous solution, pH 5) also serves as a control experiment, indicating the crucial nature of its physical state. Treatment of the nucleic acid (600 μg.) with ribonuclease (0.2 μg) in 10^{-4} *M* magnesium sulfate rendered it unable to combine with the protein to produce active rods; also, substitution of other nucleic acids (DNA from thymus and RNA from turnip yellow mosaic virus[10]) yielded almost nucleic acid-free pellets which were inactive (Table 2).

A few preliminary experiments on the properties of the reconstituted virus indicated somewhat greater lability to alkali (pH 9) than that of TMV. Ribonuclease (0.2 μg for 5 mg.) also appeared to decrease the activity. A fractionation of the nucleoprotein material by centrifugation at 25,000 rpm. for 30 minutes yielded a pellet of considerably higher infectivity than the residual half of the material, pelleted at 40,000 rpm. (1 hour). Material so purified seemed to increase in activity upon storage in 0.3–0.5 per cent solution at 3°. It also appeared to become more resistant to alkali and to ribonuclease.

Electron Microscopic Studies.—Electron microscopy was used qualitatively to study the shapes and sizes of the components involved in the formation of the virus rods and quantitatively[11] to assess the degree of homogeneity of the suspensions as well as to obtain counts and length distributions of the reconstituted rods. In the protein, the only visible particles, aggregates presumably of the original 4.5 S material, appeared as disks about 5–15 mμ thick and with central holes. The diameter of the disk was 15 mμ and that of the central hole about 4 mμ (Fig. 2).

TABLE 2

EFFECT OF REACTION CONDITIONS ON REGENERATION OF VIRUS ACTIVITY

REACTION CONDITIONS*	ASSAYS μg./Ml.	ASSAYS Lesions/Half-Leaf
Protein (1 per cent) + RNA (0.1 per cent):		
1 minute, pH 6	100	0.6
24 hours	100	10.2, 13.1†
96 hours	100	13.0
Protein (0.01 per cent) + RNA (0.001 per cent):		
24 hours, pH 6	100	0.6, 0.2†
96 hours	100	0.1, 0.2†
Protein + RNase-digested RNA	100	0.0
Protein + TYMV-RNA	100	0.3
Protein‡	500; 625; 1800	0.0; 0.0; 0.0
RNA‡	30; 52; 230	0.0; 0.0; 0.3

* Protein and RNA stand for the preparations isolated from TMV by the methods described in the text. TYMV-RNA is the ribonucleic acid isolated from turnip yellow mosaic virus. RNase is ribonuclease.

† Reassayed dilute solution after one week.

‡ Different preparations, assayed about 10 times, generally on separate plants (see n. 9); in the customary half-leaf assay method, up to 0.5 lesions were occasionally obtained with the same preparations.

The appearance of these perforated disks is identical with that of partially polymerized X-protein.[5] In the nucleic acid solution only occasional poorly defined fibrils could be detected. The reconstituted rods appeared to be identical in shape and size with intact TMV, except for a greater randomness of length (Fig. 3).

Preparations of the purified protein and nucleic acid were examined for any electron microscopic evidence of contamination with intact TMV. In a specific instance the protein was sprayed upon the specimen screens at a concentration of 0.1 per cent and the RNA at 0.007 per cent. These are tenfold and sevenfold greater concentrations of the two components than the highest levels used in the assays of the reconstituted virus (100 μg./ml.). The protein preparation showed no rods of a length even as great as 100 mμ in six droplet patterns each of which had a volume of approximately 3×10^{-9} ml. The RNA material also showed no rods in six droplets of approximately this average volume. From these figures it can be calculated that the constituent protein and RNA solutions contribute fewer than 5×10^{7} typical TMV particles per milliliter to the reconstituted virus in the

highest concentration assayed. Additional counts made on the standard TMV used as an infectivity control showed that approximately 7×10^8 particles per milliliter, of typical length, are required to produce the 10 lesions per leaf custom-

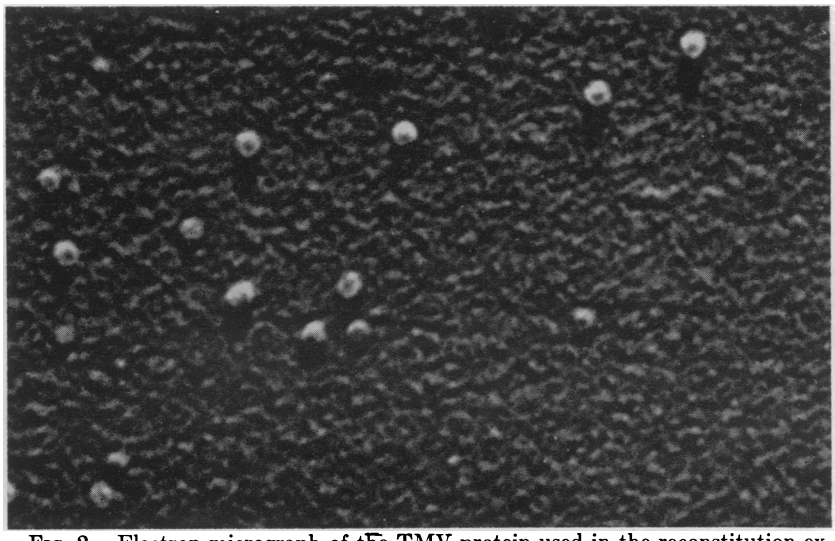

FIG. 2.—Electron micrograph of the TMV protein used in the reconstitution experiments. The particles are characteristically disk-shaped, with central holes. ×120,000.

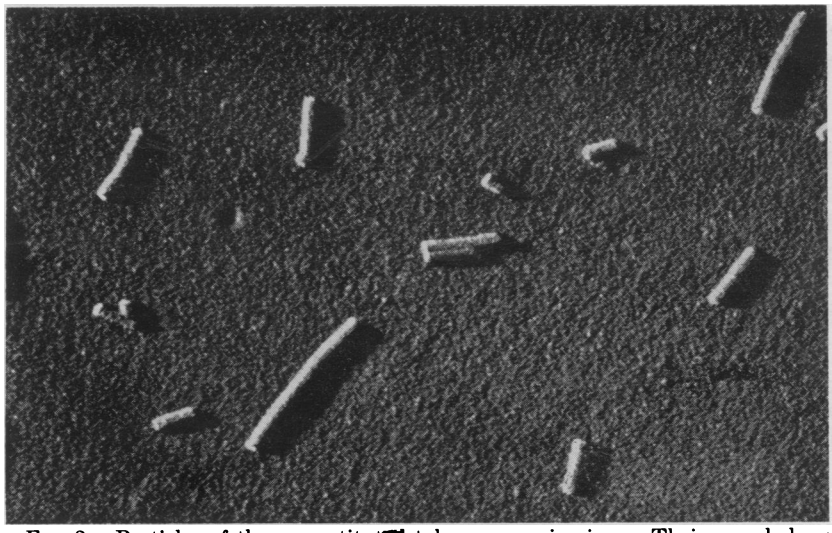

FIG. 3.—Particles of the reconstituted tobacco mosaic virus. Their morphology is identical with that of normal TMV, except for a greater proportion of short particles. The longest rod in this field is about 300 mμ long. ×60,000.

arily obtained. Inasmuch as several preparations of the reconstituted virus gave about twice that number of lesions at the 100 μg./ml. level, we can conclude that the starting materials failed by at least a factor of 30 to contain enough contaminating TMV particles to account for the final infectivity. This conclusion rests

upon the supposition that the specific infectivity of any TMV particles that might remain as contaminants in the protein and RNA preparations is the same as that possessed by the untreated control TMV.

Counts and length distributions were secured for the particles of both TMV and the most active reconstituted virus, the 25,000-rpm. pellet described above, in order to ascertain the relative biological efficiency of the two materials in terms of numbers of particles per milliliter per lesions per leaf. The results of the counts and length distributions are given in Table 3. From this table it appears that about one-tenth of the total particles in the reconstituted virus, representing about one-third of the total mass, were of length *ca.* 300 mμ, the length of the monomer of normal TMV. These particles were therefore only about 3 per cent as infective as the particles of similar length in the control TMV. It thus appears that polymerization of the protein by nucleic acid to form ~300-mμ rods is fairly frequent but that only a fraction of the rods is reconstituted with the structural faithfulness necessary for infectivity.

TABLE 3

ELECTRON MICROSCOPE COUNTS OF PARTICLES OF TMV AND RECONSTITUTED VIRUS AT ASSAY CONCENTRATION

Preparation	Assay Concentration (μg./Ml.)	Lesions/ Half-Leaf	Total Particles/Ml.	Particles/Ml. of Length 290–310 Mμ	Particles/Ml./ 10 Lesions
Control TMV	0.1	10	1.6×10^9	7.0×10^8	7×10^8
Reconstituted virus (25,000 rpm.)	10	10	2.2×10^{11}	2.0×10^{10}	2×10^{10}

The length distribution of the rods of this pelleted material exhibited a reasonably random character for lengths less than about 260 mμ, but a highly uniform length between 290 and 310 mμ for the 10 per cent of the rods falling in this range. Only 3 per cent of the particles were of lengths greater than 310 mμ. This is in contrast to aggregates of X-protein, which show a complete randomness of distribution of lengths.

To ascertain whether the nucleic acid was localized in the center of the newly formed virus rods, Dr. R. Hart applied his technique of detergent treatment, followed by electron microscopical analysis.[5] He found the reconstituted rods appreciably more labile to SDS than was standard TMV, but after SDS treatment for 10 seconds many rods were partially degraded and showed a central strand of material protruding from the ends, as does standard TMV after 60 seconds of reaction (Fig. 4). These strands disappeared when ribonuclease was added.

Summary.—The preparation from TMV of protein and RNA fractions which tend to recombine at about pH 6 to form a nucleoprotein carrying virus activity (0.1–1 per cent of that of TMV) is described.

An electron microscopic search revealed no TMV rods in either of the two component solutions at a concentration level thirty fold to three hundred fold greater than those at which the reconstituted virus was assayed. In the latter, on the other hand, up to about one-third of the material consisted of rods of the typical diameter and length of TMV, many, if not all, containing a nucleic acid core.

The concentration, time, and pH dependence of virus regeneration is that of a typical chemical reaction. Freshly prepared RNA is required for appreciable reaction; degraded RNA, or nucleic acids from other sources, are inactive.

No inhibition was observed if known small amounts of TMV were added to concentrated solutions of each of the components and subsequently diluted and assayed.

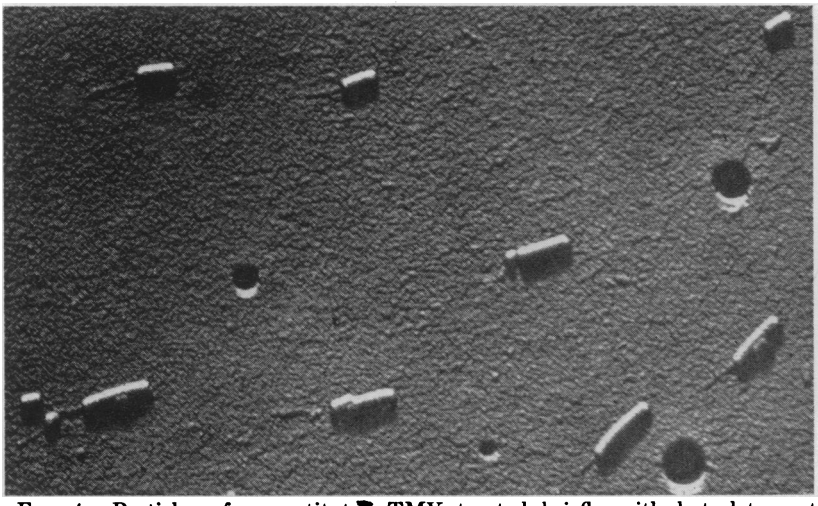

FIG. 4.—Particles of reconstituted TMV treated briefly with hot detergent. Rods are seen with strands of nucleic acid projecting from their ends. This appearance is identical with that found for normal TMV more severely treated. ×55,000.

The evidence thus seems reasonably complete that, under the conditions described, TMV nucleic acid enters into combination with TMV protein subunits and favors aggregation to rods, some of which are of sufficient length and structural integration to carry infectivity.

The capable assistance of Mrs. B. Singer and Mr. J. Toby is gratefully acknowledged.

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[7] S. S. Cohen and W. M. Stanley, *J. Biol. Chem.*, **142**, 863, 1942.

[8] When less than 6 per cent of nucleic acid is added to the protein, almost all is incorporated in the pellet, but lower infectivity is the result. Protein alone also forms pellets upon ultracentrifugation at pH 6.

[9] The usual assay method yielded usually 0.0, but at times up to 0.4, lesions per half-leaf when phosphate alone was applied. This result is probably due to accidental contamination by active material applied to other sites of the plant. Therefore, separate plants were used when the absence of active virus was in question. Standard TMV was applied to one bottom leaf

and the unknowns to all others. This gave consistently 0.0 lesions with the components used in virus reconstitution, except when the nucleic acid was applied at 200 μg./ml., twenty times the amount present at the highest assay level of the reconstituted virus (0.3 lesions per half-leaf).

[10] Kindly supplied by Dr. S. S. Cohen and Dr. V. Cosentino.

[11] R. C. Williams, R. C. Backus, and R. L. Steere, *J. Am. Chem. Soc.*, **73**, 2062, 1951.

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*ENZYME FORMATION IN PROTOPLASTS OF BACILLUS MEGATERIUM**

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INTRODUCTION

Results of the last two years[1, 2] have led rather compellingly to the conclusion that the induced synthesis of enzyme occurs via a mechanism which condenses amino acids on a template. The possibility that amino acids are "activated" prior to adsorption on the template seems likely from recent preliminary reports.[3, 4] In any event, no evidence for a stable intermediate, smaller than the fully formed enzyme, has yet emerged. If one accepts the template hypothesis, it is evident that further understanding of the mechanism of enzyme formation will not be achieved through attempts at analysis of intermediate stages of protein synthesis, since none exist. This view forces concentration on experiments designed to elucidate the nature and functioning of the template. Data obtained[2, 5] with intact cells synthesizing enzyme suggest that the template is RNA. However, the distance between the observations and the derived conclusions in such experiments is too great for certainty. The need is clear for a subcellular fraction possessing enzyme-forming ability and amenable to more directly interpretable dissection of its components and their function. The recent work of Gale and Folkes[6, 7] on ruptured cell preparations of *Staphylococcus aureus*, and of Zamecnik and Keller[8] on a liver microsome fraction, indicates that the attainment of such systems is close at hand.

Weibull's[9] observation on "protoplast" formation resulting from lysozyme treatment of *Bacillus megaterium* cells in hypertonic medium suggested that another analyzable subcellular system was available. Protoplasts are morphologically quite dissimilar from the rod-shaped cells from which they are derived, being spherical and smaller. Each rod usually yields between 2 and 3 spheres. Lester[10] and Beljanski[11] were able to show that lysozyme treated *B. megaterium* could incorporate labeled amino acids. The question remained whether protoplasts could be induced to synthesize enzyme. This potentiality has been realized. It has been found possible to devise a properly supplemented stabilizing medium, which permits protoplasts of *B. megaterium* to form beta-galactosidase at rates comparable to those of intact cells. Simultaneously, Wiame and his collaborators (personal communication) succeeded in obtaining formation of arabinokinase in lysozyme treated preparations of *Bacillus subtilis*.